SPECIES OF RNA EXTRACTED FROM TOBACCO AND DATURA PLANTS AND THEIR DIFFERENTIAL SENSITIVITY TO ACTINOMYCIN D.

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SUMMARY

RNA was extracted from leaves of tobacco with phenol and sodium dodecyl sulfate (SDS) and analyzed on sucrose gradients. A 13s RNA component was found to be associated with the light ribosomal RNA peak, shifting this peak towards the 13s region. The synthesis of this 13s RNA species is relatively resistant to actinomycin D, and is almost absent from RNA preparations extracted without SDS. A similar peak was found in sucrose gradient profiles of Datura stramonium RNA, but at the 11s region of the gradient. RNA synthesis in Datura leaves is blocked following detachment of the leaves from the plant, but the synthesis of this particular 11s RNA component does not cease, either in detached leaves or in actinomycin-treated leaves. The RNA nature of this AMD-resistant peak was established by its sensitivity to ribonuclease, by its insensitivity to deoxyribonuclease, as well as by electrophoresis and radioactive scanning of polyacrylamide gels.

INTRODUCTION

Actinomycin D (AMD) is known as a specific inhibitor of DNA-dependent RNA synthesis in bacterial and mammalian systems (1, 2, 3). However, information about its effect on RNA synthesis in higher plants is scant, as most of it was gathered indirectly from cases where AMD was used in studies concerning aspects other than cellular RNA synthesis (4, 5, 6, 7).

In the course of our virological work with AMD we came across some unexpected results which are discussed below. Most significant among these were: (a) Incomplete inhibition of RNA synthesis in leaves treated with high concentration of AMD, which is contrary to previous reports claiming virtually total inhibition (4, 7). (b) The radioactive peak of the newly synthesized RNA, corresponding to the 17s RNA species, did not precisely superimpose on the respective absorbancy peak. The following experiments were therefore devised in order to clarify these and other discrepancies.

MATERIALS AND METHODS

Water, actinomycin and uracil 2-^{14}C were administered to individual, detached leaves of tobacco (Samsun, Var. N.N.) or Datura stramonium L. through the petioles. Every leaf was held separately in a humid chamber until all the solution was taken up. The detached leaves were given 24 hours to take up the AMD solution, followed by an additional 24 hours for the uptake of the radioactive uracil solution. Extraction of RNA was thus made 48 hours after the detachment of the leaves. Actinomycin D (Serva) was applied at 5-40 ug/g leaf tissue, while ^{14}C-uracil was applied at 1uCi per leaf of Datura and 3uCi per leaf of tobacco. Tobacco RNA was labeled for gel scanning with ^{32}P orthophosphate at 8uCi/leaf.

Ten g of frozen leaves were homogenized in a Sorvall Omnimixer at half speed for 2 minutes with a mixture containing 20 ml of water-saturated phenol, 9 ml of glycine buffer (0.14 M glycine, 0.1 M NaCl, 0.01 M EDTA, pH 9.5), 1 ml of 10% bentonite, prepared according to Fraenkel-Conrat et al. (10) and 1 ml of 10% sodium dodecyl sulfate (SDS). After low speed centrifugation the upper layer was collected and treated again with phenol. RNA was precipitated twice with 2 volumes of ethanol, washed with ether, redissolved in a small volume of water, and 15 optical density units of it

were applied to the top of 5-20% linear sucrose gradients in the above glycine buffer. Gradients of 12.5 ml were run at 30,000 rpm in type SB-269 rotor of the B-35 International ultracentrifuge for 8.5 hrs at 5°. Fractions, 0.5 ml each, were collected by means of a polystatic pump and their absorbancies were read at 260 mu. Radioactivity of the same fractions was counted in a Packard liquid scintillation fluid (fluors dissolved in 7:3 v/v of toluene:2-ethoxyethanol). Since the main interest was focused on the vicinity of the 17s peak, no attempts were made to remove unspecified substances of low molecular weight which in some cases accumulated on the top of the gradients.

Polyacrylamide gels (3%) were prepared and electrophoresed exactly as described by Loening (8). At the end of the run, gels were extruded from their tubes and UV absorbance profiles were recorded with a gel scanner mounted on a Simadzu QV50 spectrophotometer constructed by Gressel and Wolowelsky (9). The gels were then placed on a strip of filter paper and dried flat under an infra-red lamp. The dried gels were scanned for radioactivity with the Packard Model 7201 Radiochromatogram Scanner.

The RNA preparations probably contained some residual nucleases, since incubation at 37° for 30 minutes resulted in their complete degradation. Hence, the RNA preparations were treated with diethylpyrocarbonate (DEP) before testing for sensitivity to ribonuclease (RNase) or deoxyribonuclease (DNase) as follows:

The RNA preparation was incubated with 30 ul/ml of DEP for 30 min at room temperature. Under such circumstances even added RNase or DNase were completely inactivated. The DEP was self-hydrolyzed overnight at 4°, and the RNA was precipitated with ethanol. The obtained preparation was free from active nucleases as well as from interfering amounts of DEP. This was indicated by the stability of the DEP-treated

preparation during incubation at 37°, and the non-inhibited activity of newly added RNase or DNase to the above preparation.

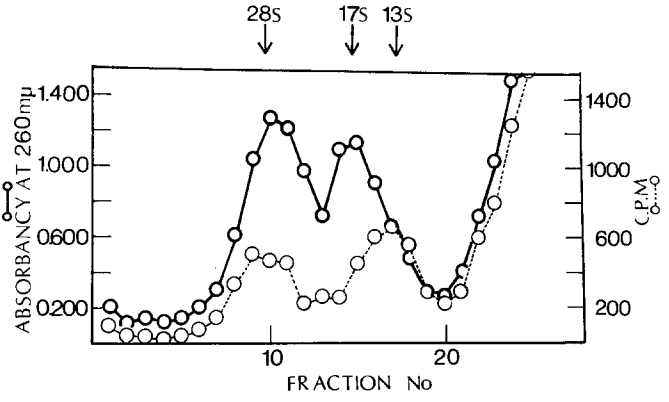

Fig. 1: Sedimentation profiles of tobacco RNA labeled by ^{14}C-uracil and extracted by the phenol-SDS method.

RESULTS AND DISCUSSION

RNA was extracted from tobacco leaves, untreated with AMD, after uptake of ^{14}C-uracil and was run on sucrose gradients as described above (Fig. 1). The same procedure was employed for AMD-treated leaves (Fig. 2) and for AMD-treated leaves from which the RNA was extracted without the use of SDS (Fig. 3). In general, the above figures exhibit the expected inhibitory effect of AMD on RNA synthesis, as demonstrated by the reduction in the synthesis of the two ribosomal RNA species. Suppression of RNA synthesis is almost complete in preparations extracted without SDS (Fig. 3). However, the use of this detergent apparently helped to release an additional species of RNA, positioned at the 13s region, which is resistant to AMD (Fig. 2). This, in turn, led to inhibition rates of RNA synthesis which are lower than those reported previously (4, 7), being sometimes as low as 50%. A close study of the radioactive profiles of the newly synthesized RNA in control leaves (Fig. 1) reveals

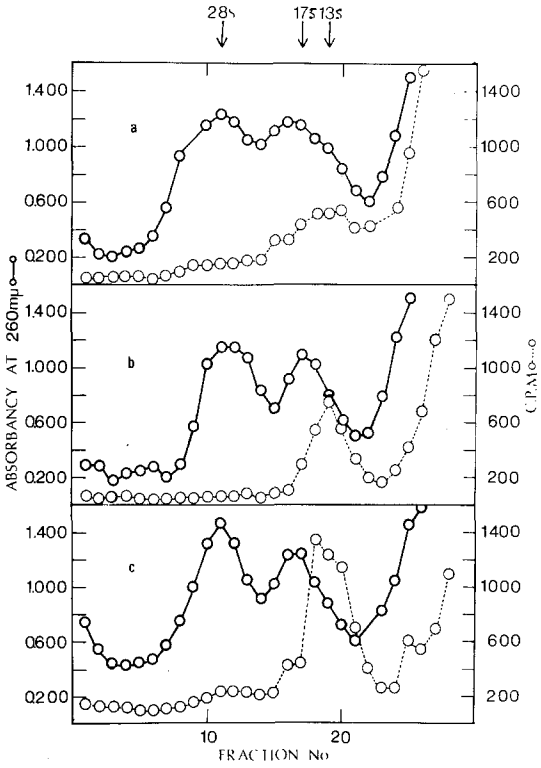

Fig. 2: Sedimentation profiles of tobacco RNA extracted from leaves treated with different concentrations of AMD. (a) 40 ug AMD per 1g leaf; (b) 20 ug AMD per 1g leaf; (c) 5 ug per 1g leaf.

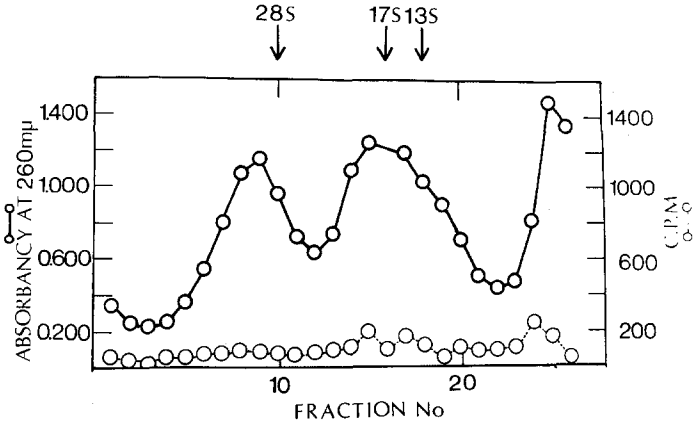

Fig. 3: Sedimentation profiles of RNA extracted from AMD-treated tobacco leaves without SDS.

a shift in the position of the 17s peak towards the 13s region. Hence, it seems that also in leaves untreated with AMD, the light ribosomal RNA peak is composed of two different RNA species, namely a ribosomal RNA and a lighter species which is AMD resistant. Moreover, it appears that the synthesis of the 13s RNA is enhanced by low doses of the antibiotic and only slightly suppressed by increasing amounts of AMD (Fig. 2). In this connection it appears that the previously reported differential AMD-sensitivity of the two ribosomal RNA species (11) could be attributed to the association of an additional RNA species with the light ribosomal RNA peak, similar to the above-reported 13s RNA. This is in line with the widely accepted model of a 45s parent molecule for both ribosomal RNA species, transcribed as a single unit on the same cystron (12). If the site of inhibition of RNA synthesis by AMD is indeed the DNA template, then, according to the above model, AMD is unlikely to differentially inhibit the two ribosomal RNAs. The RNA nature of the 13s peak was demonstrated by its sensitivity to ribonuclease as follows:

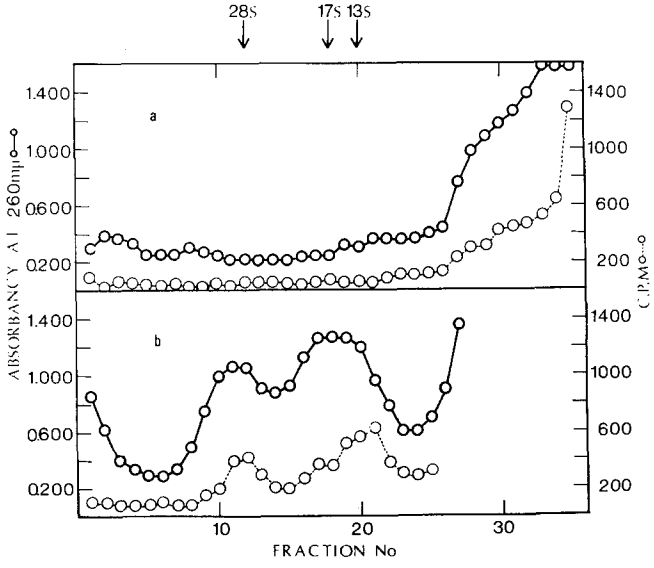

Fig. 4: Sedimentation profiles of: (a) RNA from AMD-treated tobacco leaves, digested with RNase; (b) the same as (a) but with no RNase digestion.

Extracted RNA was treated with DEP, washed with ethanol, dissolved in SSC buffer (0.14 M NaCl, 0.01 M sodium citrate, 0.01 M $MgCl_2$, pH 7.2) and incubated with 10ug/ml of pancreatic ribonuclease at 37° for 30 min prior to centrifugation in sucrose gradient made in the above SSC buffer (Fig. 4).

The distribution of RNA from Datura showed a pattern similar to that of tobacco RNA (Fig. 5c). However, RNA synthesis virtually ceased in detached Datura leaves, and the picture after 72 hours resembled that of AMD-treated leaves (Fig. 5b). The RNA of Datura also exhibited an AMD-resistant peak, except that this peak is positioned in the

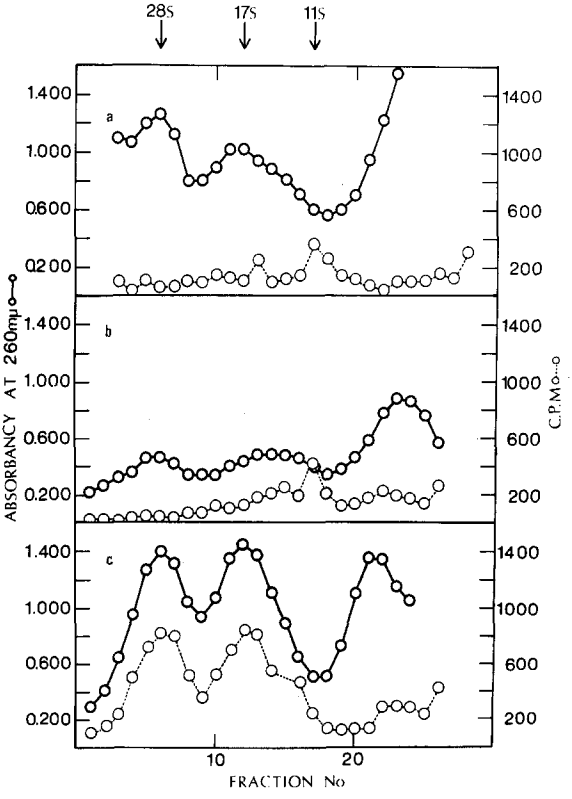

Fig. 5: Sedimentation profiles of RNA from Datura leaves treated as follows, prior to the 24-hr labeling period: (a) dipping in AMD solution (20 ug/g leaf) for 48 hrs; (b) dipping in water for 48 hrs; (c) dipping in water for 16 hrs.

lls region rather than the 13s of its tobacco analogue, and was resistant also to the combined effect of AMD and detachment of the leaf (Fig. 5a). Interestingly enough, the synthesis of the lls RNA went on unaffected by the mechanisms which suppress the majority of the other RNA species.

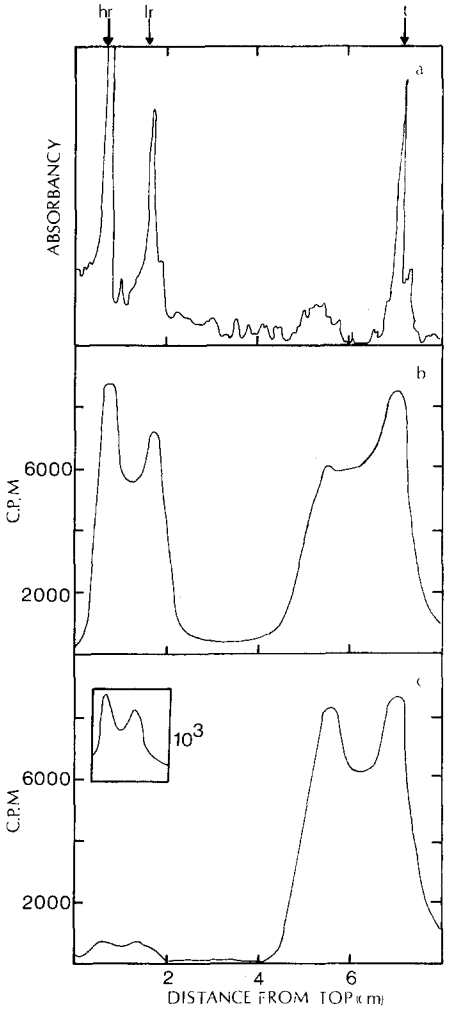

Fig. 6: Patterns of ^{32}P-labeled tobacco RNA electrophoresed on polyacrylamide gels. Section "a" represents UV scanning. Sections "b" and "c" represent strip scanning of radioactivity of RNA; (b) extracted from untreated leaves; (c) extracted from AMD-treated leaves. In the inset of section "c" the AMD-inhibited ribosomal RNAs are plotted on an expanded scale.

^{32}P-labeling, gel electrophoresis and DNase-insensitivity were used as additional criteria to prove the RNA nature of the AMD-resistant peak. RNA from tobacco was labeled with ^{32}P and extracted as described above. The labeled RNA (37 ug, 259,000 cpm) was applied to the top of the gel and electrophoresed for 110 min at 5mA per gel. Gels were then scanned for UV absorbance and radioactivity as described before. Fig. 6 (b, c) illustrates the radioactivity pattern obtained with RNA extracted from untreated and AMD-treated leaves. Again, the synthesis of the two ribosomal RNA species was substantially blocked, whereas that of the tRNA

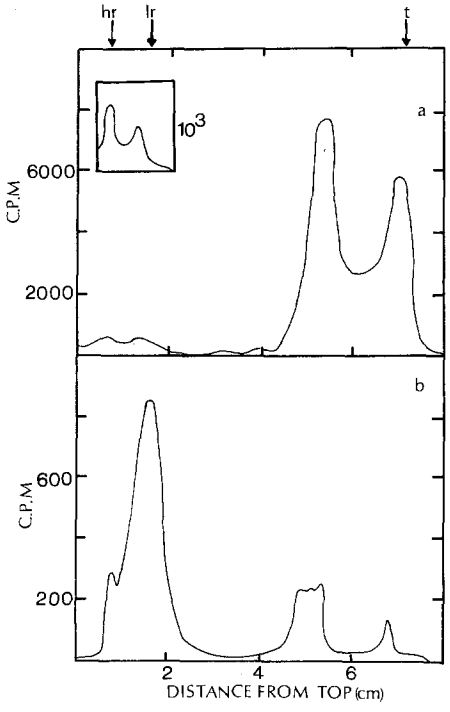

Fig. 7: Patterns of ^{32}P-labeled tobacco RNA electrophoresed on polyacrylamide gels. (a) RNA from AMD-treated leaves as in Fig. 6c, but incubated with DNase prior to electrophoresis. (b) light-ribosomal RNA fractions obtained from sucrose gradient following extraction from AMD-treated leaves. In the inset of section "a" the ribosomal RNAs are plotted on an expanded scale. Note that the scale range of section "b" is also expanded.

was only slightly affected. However, a radioactive band, which is AMD-resistant, appeared between the light ribosomal RNA and the tRNA bands. In fact, the possibility that this additional peak may be DNA can be ruled out merely by its position on the gel. Nevertheless, DNase was applied to a DEP-treated RNA preparation prior to electrophoresis in order to verify the above assumption. The AMD-resistant peak was indeed found to withstand DNase digestion (Fig. 7a).

Both AMD-resistant peaks of the sucrose gradient and of the acrylamide gel seem to be identical, as indicated by the following test: ^{32}P-labeled RNA extracted from AMD-treated tobacco leaves was run on sucrose gradient as described above. The fractions of the light ribosomal RNA and a few neighboring fractions in each direction, were pooled together. Carrier tRNA (E. coli) was added, and RNA was precipitated in ethanol. This RNA was then electrophoresed in the usual manner (Fig. 7b). The major radioactive band was the expected light ribosomal RNA. Residues of the heavy ribosomal RNA and the tRNA could also be observed (note that the cpm range of section "b" in Fig. 7 is the same as that of the inset in section "a" of that Figure). The extra peak, attributed to the AMD-resistant RNA, also appeared at the expected position. However, its intensity was lower than expected, probably because of incomplete precipitation from the low concentration of RNA in the sucrose solution.

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